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The metabolism of β-chloroprene: preliminary in-vitro studies using liver microsomes

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Abstract

Based on analogy with butadiene and isoprene, the metabolism of β-chloroprene (2-chloro-1,3-butadiene, CD) to reactive intermediates is likely to be a key determinant of tumor development in laboratory rodents exposed to CD by inhalation. The purpose of this study is to identify species differences in toxic metabolite (epoxide) formation and detoxification in rodents and humans. The in-vitro metabolism of CD was studied in liver microsomes of B6C3F1 mice, Fischer/344 and Wistar rats, Syrian hamsters, and humans. Microsomal oxidation of CD in the presence of $NADP^+$, extraction with diethyl ether, and analysis by GC-mass selective detection (MSD) indicated that (1-chloroethenyl)oxirane (CEO) was an important metabolite of CD in the liver microsomal suspensions of all species studied. Other potential water-soluble oxidative metabolites may have been present. The oxidation of CD was inhibited by 4-methyl pyrazole, an inhibitor of CYP 2E1. CEO was sufficiently volatile at 37°C for vial headspace analysis using GC-MSD single ion monitoring ($m/z = 39$). CEO was synthesized and used to conduct partition measurements along with CD and further explore CEO metabolism in liver microsomes and cytosol. The liquid-to-air partition coefficients for CD and CEO in the microsomal suspensions were 0.7 and 58, respectively. Apparent species differences in the uptake of CEO by microsomal hydrolysis were hamster ~ human > rats > mice. Hydrolysis was inhibited by 1,1,1-trichloropropene oxide, a competitive inhibitor of epoxide hydrolase. A preliminary experiment indicated that the uptake of CEO in liver cytosol by GSH conjugation was hamster > rats ~ mice (human cytosol not yet tested). In general, the results suggest that metabolism may help explain species differences showing a greater sensitivity for CD-induced tumorigenicity in mice, for example, compared with hamsters. Additional experiments are in progress to quantify the kinetic parameters of

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CD oxidation and CEO metabolism by enzymatic hydrolysis and conjugation by glutathione *S*-transferase for in cytosol. A future goal is to use the kinetic rates to parameterize a physiologically based toxicokinetic model and relate the burden of toxic metabolite to the cancer dose–response observed in experimental animals.

Keywords: 2-Chloro-1,3-butadiene; (1-Chloroethenyl)oxirane; Metabolism; Species differences

1. Introduction

β-Chloroprene (2-chloro-1,3-butadiene, CD, CAS 126-99-8) is a colorless volatile liquid almost exclusively used to manufacture polychloroprene rubber [1]. Human exposure can occur primarily by inhalation during the manufacturing and shipping of CD monomer, and during the polymerization process [2]. The epidemiology data have been reviewed and are insufficient to conclude that CD is carcinogenic in humans [3–5]. A new study of CD epidemiology is under way [6].

Concern for potential adverse effects of CD in humans has led to numerous studies of toxicity in experimental animals (reviewed in Refs. [3,7,8]). Scientific interest recently increased following the results of chronic inhalation studies in mice and rats showing that CD is a multi-site carcinogen. CD was tumorigenic in male and female B6C3F1 mice and Fischer/F344 rats exposed to concentrations of 0, 12.8, 32, or 80 ppm (6 h/day, 5 days/week, for up to 104 weeks) [9]. Tumors in mice (male and female) were observed in the lung, circulatory system, Harderian gland, and forestomach. Tumors were also observed in the kidney (male mice only) and mammary gland, skin, mesentery, Zymbal gland, and liver (female mice only). Tissues affected in Fischer rats included the oral cavity, thyroid gland, and kidney (male and female), lung (male only) and mammary gland and kidney (female only) [9]. In contrast to the clear evidence of tumorigenicity in the B6C3F1 mice and Fischer rat, a chronic inhalation study in which Wistar rats and Syrian hamsters were exposed to 0, 10, and 50 ppm (6 h/day, 5 days/week, 24 months for rats and 18 months for hamsters) showed no clear evidence of tumorigenicity. In the Wistar rat, a weak response for mammary gland fibroadenoma was reported at 50 ppm. No tumors were found in the hamster [9,10].

The genetic toxicology was recently reviewed and indicated conflicting results dependent on the method of testing [8]. In general, the in-vitro studies suggest that CD is mutagenic, primarily in bacterial reverse mutation assays [11–17], while other in-vitro and in-vivo assays for either gene mutation or structural chromosomal damage were negative [11,18–22]. Possible factors influencing the outcome of the in-vitro mutagenicity studies included the exposure method (plate incorporation or pre-incubations), the purity of CD and presence of dimers or peroxides produced in aged CD, and the presence or absence of metabolic activation system during incubation [8].

The purity of CD and methods of exposure are important factors affecting the interpretation of the CD-induced genotoxicity and tumorigenicity. Metabolism is another especially important factor in understanding the results of the chronic

bioassay, and species and target organ differences in susceptibility. The formation and detoxification of epoxide metabolites are well established as contributing factors in the toxicity and tumor induction by 1,3-butadiene and isoprene [23]. A number of reports (from 1950 to 1973) suggested a variety of effects of CD on biochemical and metabolic endpoints primarily associated with liver function (see summary by Haley [24]); however, insufficient experimental details hinder the interpretation of these reports. The few available metabolism studies provide limited evidence that CD is a substrate for oxidation by microsomal mixed function oxidases. Bartsch et al. [13] showed that an unidentified volatile metabolite could be trapped with 4-(4-nitrobenzyl)pyridine and suggested the formation of epoxide intermediate(s). At that time, the metabolism of CD was proposed to be similar to that of vinyl chloride's metabolism to a reactive epoxide [8,24]. Indirect evidence for involvement of GSH in the metabolism of CD was reported [25,26]. Rats that were fasted and exposed to CD by inhalation had a greater hepatotoxicity compared with non-fasted rats. Further studies in rats showed a decrease in hepatic non-protein sulfhydryl content after administration of a single oral dose of CD (100–200 mg/kg) [27]. The urine of these rats was found to contain thioethers that were postulated to represent GSH-conjugates or mercapturic acid metabolites of CD.

The goal of this research is to further explore the metabolic pathways for CD. The expectation is that metabolic differences in the fate of CD in rodents will help explain the differences in species sensitivity observed in the inhalation bioassays, and the findings will be useful in human risk assessment. The research presented here describes the application of in-vitro metabolism techniques to identify the cytochrome P450-dependent formation of a reactive epoxide metabolite of CD in liver microsomes. One important epoxide metabolite was identified as (1-chloroethenyl)oxirane (CEO), and further in-vitro experiments were conducted to investigate the potential for species differences in CEO detoxification by microsomal hydrolysis and GSH-mediated conjugation in cytosol.

2. Materials and methods

2.1. Chemicals

β-Chloroprene (CD, $>99\%$ purity) inhibited with phenothiazine and *N*-nitrosodiphenylamine, was supplied by DuPont-Dow Elastomers L.L.C. (LaPlace, LA). Special care was used for handling and storage because dimerization, oxidation, and auto-polymerization of liquid CD will occur if exposed to air at room temperature. The inhibitors were removed prior to study by passage through activated alumina (D-37269, ICN Biomedicals, GmbH, Germany) under a N_2 atmosphere. The CD was collected into glass vials, sealed with silicone–Teflon®-lined septa and stored in an air-tight metal can at < -70°C. Opened vials were re-sealed with a blanket of N_2. CD stored under these conditions has remained $>98\%$ pure for $2+$ years based on analysis by gas chromatography-flame ionization detection.

Other chemicals used: 1-butanol, 3,4-dichloro-1-butene, 3-chloro-benzenecarboperoxoic acid, dichloromethane, potassium hydroxide (KOH), disodium ethylenediaminetetraacetate (EDTA), glucose-6-phosphate (G-6-P), G-6-P dehydrogenase, glutathione (reduced GSH), magnesium chloride, $NADP^+$, mono sodium phosphate, sucrose, TRIZ buffer, and 4-methylpyrazole hydrochloride (4-MP) were from Sigma-Aldrich Company. 1,1,1-Trichloropropene oxide (TCPO, CAS 3083-23-6) was from Maybridge Chemical Company (Trintagel, Cornwall, UK). Diethyl ether (Burdick & Jackson High Purity Solvent) was purchased from VWR Scientific Products.

2.2. Preparation of microsomes and cytosol

Microsomes and cytosol were prepared by differential centrifugation [28,29] of livers pooled from male B6C3F1 mice, Fischer 344 rats, Wistar rats, or Golden Syrian hamsters (Charles River Laboratories, Raleigh, NC). The cytosolic fraction was saved, and the microsomal pellet was washed with, and suspended in, 0.25 M sucrose, 50 mM Tris, 5.4 mM EDTA, pH 7.4. The cytosolic and microsomal fractions were stored at < -70°C. Protein concentrations were measured using the Bio-Rad Protein Assay Kit, based on the Bradford dye-binding procedure. Human liver microsomes were purchased as a mixed pool from 15 different individuals (Lot #1001, In Vitro Technologies, Baltimore, MD); donor demographic information and metabolic characterization was available but not presented for the sake of brevity.

2.3. Synthesis, identification and metabolism of CEO

2.3.1. Synthesis of CEO

The (1-chloroethenyl)oxirane (CEO, CAS 3132-77-2, bp 110°C) was synthesized according to published methods [30,31] by oxidation of 3,4-dichloro-1-butene with 3-chloro-benzenecarboperoxoic acid to form the intermediate 3,4-dichloro-1-epoxybutane. The intermediate was de-chlorinated under a N_2 atmosphere by dropwise addition to molten KOH (at 120°C), resulting in the flash distillation of product and collection of the condensed vapors at room temperature. A second distillation under N_2 at 110°C gave approximately 75% chemical yield of CEO. The purity was $>98\%$, as determined by both GC/MSD and proton/^{13}C-NMR (data not shown).

2.3.2. Identification of CEO

Qualitative identification of CEO as an oxidative metabolite of CD was conducted using incubation methods similar to those published by Csanády et al. [28]. Screw-cap vials (empty volume 25.26 ml based on gravimetric calibration with water) with Teflon®-coated silicone septa were prepared containing microsomal suspensions (2 ml) consisting of 0.1 M phosphate buffer, $MgCl_2$ (15 mM), EDTA (0.1 mM), glucose-6-phosphate (0.8 mM), and glucose-6-phosphate dehydrogenase (1 U/ml). Preliminary experiments were conducted to establish linear relationships

between the rate of metabolism and duration of the incubation (0–60 min) and microsomal protein concentrations (0.5–2 mg/ml) in Fischer rat and B6C3F1 mouse microsomes suspensions (data not shown). Subsequent experiments with all species were conducted for 30 min using 1 mg of microsomal protein/ml of suspension. The suspensions (one vial per species) were heated and agitated (37°C for 5 min) in a vortex shaker (Buchler Instruments, Lenexa, KS). The vial headspace pressure was equalized with room air, and a volume of headspace was removed and replaced with an equal volume of concentrated CD gas prepared in a Tedlar® bag (SKC Inc., Eighty Four, PA). The initial headspace concentration was 800 ppm. Reactions were initiated after a 5 min pre-equilibration with CD by the addition of $NADP^+$ (0.4 mg/ml of suspension). Control incubations were performed without $NADP^+$.

After 30 min, the reactions were stopped by the addition of cold diethyl ether containing 1-butanol as an internal standard. The ether extracts were concentrated approximately 20-to-1 and analyzed by gas chromatography mass spectroscopy (HP7683A series injector and auto-sampler, and HP6890 gas chromatograph, HP5973 MSD mass selective detector, Hewlett-Packard, Wilmington, DE). The injection volume was 1 μl. The GC/MSD was operated in the cool on-column splitless injector mode. An initial oven temperature of 30°C was held for 4 min, heated to 220°C at a rate of 10°C per minute, and held again for 5 min. The injector inlet was set to track the oven temperature by + 3°C. The column flow rate was approximately 5.0 ml/min through a deactivated capillary guard column (1 m × 0.25 mm id) and a capillary column (DB-5ms, 30 m × 0.25 mm 1.0 μm film thickness, J&W Scientific, Folsom, CA). The MSD was scanned from 29 to 450 Da in 1 s intervals in the electron impact (EI) ionization mode.

Additional experiments were conducted to establish that the oxidation of CD could be inhibited with 4-MP, a specific inhibitor of cytochrome P450 CYP 2E1 [32,33], and to detect volatile metabolites using similar incubation conditions and manual injection of headspace samples onto the GC/MSD. For the inhibition experiment, the time course of CD at a single starting concentration of ~50 ppm was determined in the presence of 1 mg of microsomal protein/ml, and $NADP^+$, with or without 4-MP (100 μM). Phosphate buffer was used as the negative control. Headspace samples (100 μl) were taken at 1, 4, 8, 12, 16, 20, and 24 min. For the detection of volatile metabolites, CD at a single starting concentration of 100 ppm was incubated with B6C3F1 mouse liver microsomes with or without $NADP^+$. Headspace samples were taken at multiple times up to 35 min after the addition of $NADP^+$. The samples were analyzed using the GC/MSD headspace method described below.

2.3.3. Metabolism of CEO by microsomal hydrolysis or cytosolic GSH conjugation

The potential for species differences in the in-vitro metabolism of CEO was compared with liver microsomes from the five animals and liver cytosol from the four rodent species. The microsomal incubation conditions were similar to those used to identify CEO, except that 10 ml vials (empty calibrated volume 11.9 ml) were used instead of 25 ml vials. The volume of the suspension was reduced to 1.0

ml, and the reaction was started by the addition of microsomal protein rather than $NADP^+$. CEO vapor was prepared in a Tedlar® bag and added to the vial to give an initial headspace concentration of 100 ppm (e.g. 400 μl of 2740 ppm CEO added to 10.9 ml vial headspace). The microsomal protein was 1 mg/ml based on preliminary experiments in which the microsomal protein concentration was varied from 0.125 to 3 mg/ml. Control incubations were conducted with boiled microsomes in phosphate buffer or phosphate buffer alone. Headspace samples (800 μl) were taken at 1, 7, 13, 19, 25, and 31 min after the addition of the microsomal protein. An experiment was conducted to establish that hydrolysis was mediated by microsomal epoxide hydrolase using TCPO (0 to 3 mM), human liver microsomes (0.5 mg of protein/ml), and CEO (2000 ppm). For this experiment, headspace samples (200 μl) were taken at 0, 12, 24, 30, 36, 48 and 60 min after the addition of microsomal protein.

Incubation with B6C3F1 mouse, Fischer rat, Wistar rat, and Syrian Hamster liver cytosol was conducted with CEO (100 ppm) in 0.1 M phosphate buffer to assess the potential for reaction with GSH. The incubation conditions were similar to those used to measure CEO hydrolysis except that cytosolic protein (1 mg/ml) and GSH (10 mM) were used. Control incubations were conducted using boiled cytosol in phosphate buffer or phosphate buffer alone. Headspace samples (800 μl) were taken at 2, 8, 14, 30, 26 and 32 min after the reactions were started by the addition of GSH and analyzed by GC/MSD as described below.

2.4. Headspace analysis of CD and CEO

The concentration of CEO in the headspace was quantified by GC/MSD. Sample injections were made using a 1 ml gas-tight syringe and the automated Gerstel Multipurpose Sampler MPS2 and temperature controllable injection CIS3 inlet (Gerstel US, Baltimore, MD). The HP6890 GC was operated with a split ratio of 20:1 and the following temperatures: inlet (250°C), oven (100°C), and MSD transfer line (280°C). The carrier gas was helium with a total flow of 53.8 ml/min and split flow of 48.9 ml/min. The column flow was approximately 1.2 ml/min through a DB-5ms capillary column, 30 m × 0.25 mm id and 1.0 μm film thickness (J&W Scientific, Folsom, CA). The retention times of CD and CEO were approximately 1.6 and 2.2 min, respectively. The CD and CEO headspace concentrations (μM) were measured by single ion monitoring of m/z 88 and m/z 39, respectively. The MSD temperatures were set at 150°C (quadrapole) and 230°C (source). Calibrations were performed by injecting known concentrations of CEO prepared in Tedlar® bags. The response was analyzed by linear regression for CEO concentrations ranging from 0.01 to 5000 ppm (0.0004 to 200 μmol/l).

2.5. Liquid-to-air partitioning of CD and CEO in liver microsomal suspensions

Partition coefficients were determined using a modification of a described tonometry method [34–36]. CD and CEO in separate sets of vials were equilibrated at 37°C for 2 h with inactivated Wistar rat liver microsomal suspensions (1 ml in

11.9 ml sealed vials) at two concentrations (5 and 200 ppm). The headspace samples (200 μl) were analyzed at 2 h using the GC/MSD method described above and calibration curves for vapors prepared in Tedlar® bags. The liquid suspensions were immediately transferred to a second set of vials using a gas-tight syringe. These vials were re-equilibrated for an additional 2 h at 37°C, and headspace samples were analyzed by GC/MSD. The amount of analyte in the headspace of the second vial was derived solely from the liquid of the first vial. Calibrations for the re-equilibration vials were performed using a third set of vials that were incubated with boiled microsomes and known amounts of CD or CEO (e.g. 0.4–11 nmol per vial). The partition coefficients, as the ratio of the liquid-to-air concentrations, were calculated for 18 vials for each analyte.

3. Results

3.1. Identification of CEO

Incubation of CD with B6C3F1 mouse, Fischer rat, Wistar rat, hamster, or human liver microsomes resulted in an apparent GC-MSD total ion current peak eluting later than CD that was not present in the absence of $NADP^+$. The retention time was 7.3 min, as indicated in the representative chromatogram obtained from

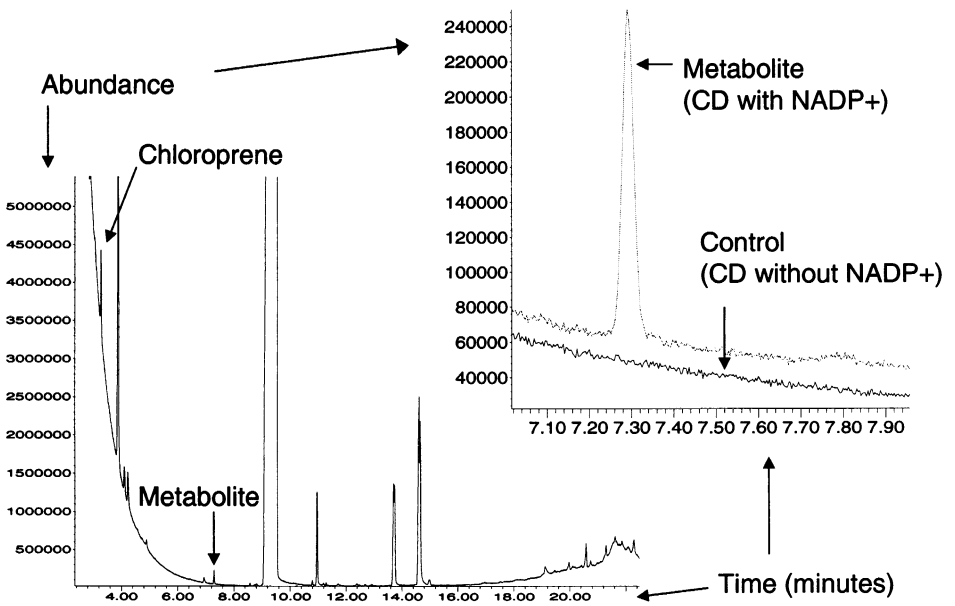

Fig. 1. GC/MS full scan total ion current for diethyl ether extract of Fischer rat liver microsomes incubated with CD. The unlabeled peaks in the full scan extract (bottom left panel) were also present in the control extract without $NADP^+$ (not shown except for the expanded upper right panel).

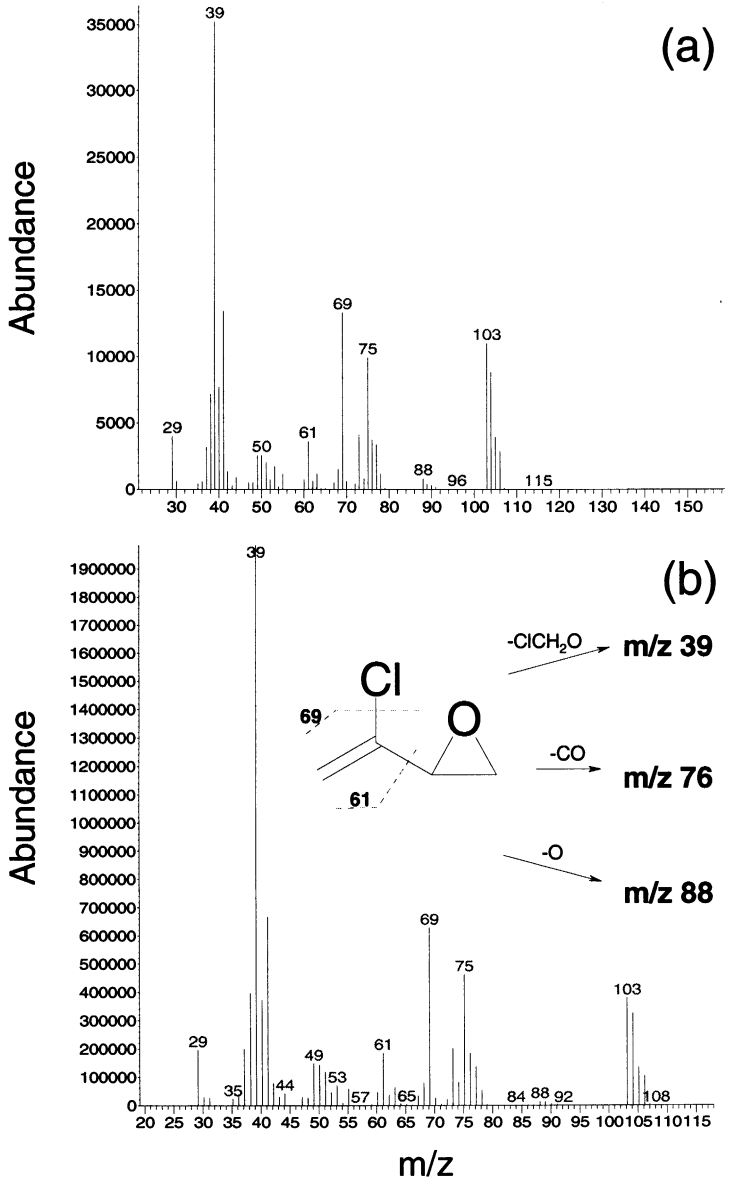

Fig. 2. Mass spectra of (a) organic extract of a reaction of Fischer rat liver microsomes with CEO, (b) authentic CEO standard.

incubation of CD with Fischer rat liver microsomes (Fig. 1). The mass spectrum for this peak was consistent with synthesized CEO standard (Fig. 2). Incubations of CD with the five different liver microsomes showed CEO to be the major peak by single ion monitoring of m/z of 39 (Fig. 3) and by full scan analysis (data not

shown). The peaks labeled 2–5 had insufficient signal to obtain meaningful spectral data. A tentative spectral match for peak 6 was made to 3-chloro-2-butenal (CAS 1679-41-0). Because peak 6 appears to be a minor metabolite relative to peak 1 (CEO) and would require an authentic standard for confirmation, no further effort was made to confirm it. The addition of 1-butanol as an internal standard to the diethyl ether extraction solvent provided for a relative comparison of the amount of CEO present in each of the five animal liver microsomes (Table 1). The analyte-to-internal standard ratio suggested that the amount of CEO present was greatest in the Fischer rat ~ B6C3F1 mouse > Wistar rat > humans ~ hamster.

Additional experiments under similar incubation conditions showed that the oxidation of CD was inhibited by 4-MP. Pretreatment of Fischer rat liver microsomes with 100 μM 4-MP showed nearly complete inhibition of CD metabolism (Fig. 4). CEO was sufficiently volatile for vial headspace analysis using GC-MSD single ion monitoring ($m/z = 39$). A decline of the CD headspace concentration from approximately 3 to 0.1 μM showed a concurrent increase of CEO in the headspace that reached a maximum of about 0.01–0.02 μM between 5 and 10 min after the start of the incubation with 100 ppm CD. The CEO headspace concentration then declined to less than 0.002 μM by 25 min (Fig. 5).

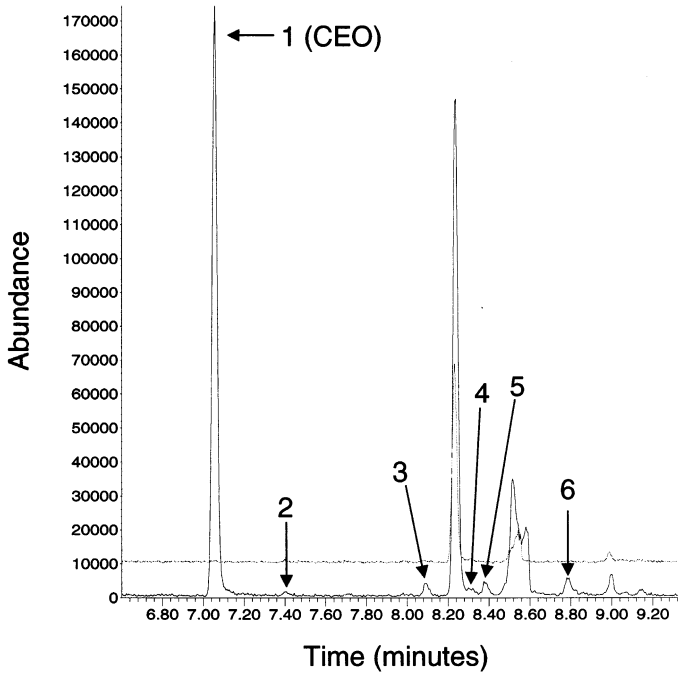

Fig. 3. Example of GC/MS single ion monitoring chromatogram (m/z 39) for Fischer rat liver microsomes incubated with CD, extracted with diethyl ether, and concentrated (bottom trace) and control incubation without $NADP^+$ (top trace). Similar chromatograms were obtained with B6C3F1 mouse, Wistar rat, hamster, and pooled human liver microsomes. Peak 1 was identified as CEO. See Table 1 for quantification of areas and text for explanation of Peaks 2–6.

Table 1
Liver microsomal metabolites as a percentage of 1-butanol internal standard[a]

Metabolite[b]	Liver microsomal suspension[c]				
	B6C3F1 mouse	Fischer rat	Wistar rat	Hamster	Human
1 (CEO)	9	12	4	0.8	1.3
2	0.0	0.1	0.1	0.2	0.1
3	0.8	0.3	0.2	0.8	0.3
4	0.2	0.0	0.1	0.4	0.1
5	0.2	0.3	0.0	0.1	0.0
6	0.6	0.4	0.3	0.3	0.1

[a] Metabolite area (m/z 39)/internal standard area (m/z 39) × 100%.
[b] Metabolite peaks are labeled in Fig. 3.
[c] One vial for each species was incubated with 800 ppm CD (0.8 μmol) for 30 min followed by diethyl ether extraction, 20-fold concentration, and cold on-column injection.

3.2. Metabolism of CEO

3.2.1. Microsomal hydrolysis of CEO

The in-vitro uptake of CEO from the vial headspace was measured at a single starting concentration (100 ppm) for each of the five animal microsomes (at 1 mg of protein/ml). The results suggest that the rate of CEO hydrolysis is hamster ~ human > rat > mouse (Fig. 6). Control incubations conducted with boiled microsomes in phosphate buffer or phosphate buffer alone showed no

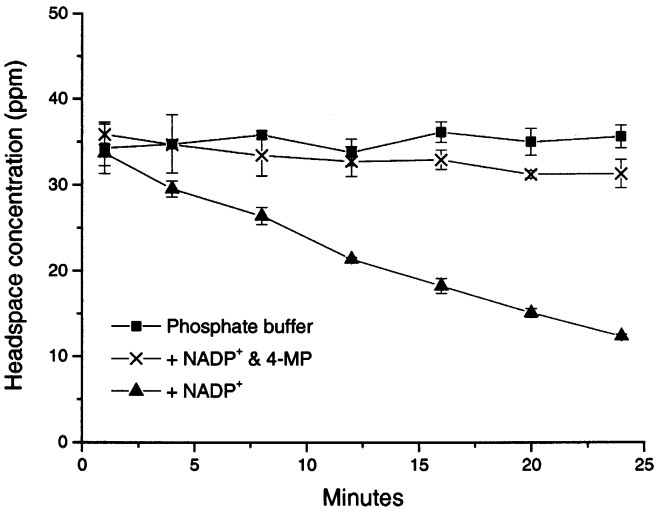

Fig. 4. Inhibition of CD (0.02 μmol) metabolism in Fischer rat liver microsomes treated with 100 μM 4-methyl pyrazole (4-MP). Values are means ± 1 S.D. for three vials per treatment. The incubation conditions are described in the text.

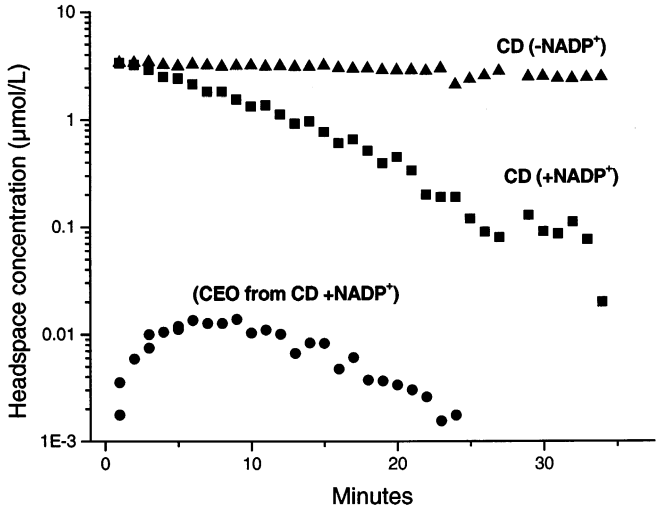

Fig. 5. Time course of CD and detection of (1-chloroethenyl)oxirane (CEO) in the headspace of B6C3F1 mouse liver microsomes incubated with 100 ppm CD (0.05 μmol).

decline in the headspace concentration of CEO (data not shown), indicating that metabolism was dependent on the presence of microsomal protein. An experiment using 0.3 mM TCPO fully inhibited the in-vitro hydrolysis of CEO (Fig. 7).

3.2.2. Conjugation of CEO with glutathione

Uptake of CEO was observed in hepatic cytosol from mice, rats, and hamsters. The rate of metabolism appeared to be fastest in hamsters compared with mice or rats (Fig. 8). Uptake nearly was absent in incubations with "cytosol–GSH" or "boiled cytosol + GSH" indicating that the reaction of CEO with GSH was enzyme-dependent. Human liver cytosol has not been tested yet.

3.3. Partition coefficients for CD and CEO in liver microsomes

The liquid-to-air partition coefficients were quantified to relate the concentration of CD or CEO in the headspace to the concentration in the microsomal suspension. The partition coefficient for CD was 0.69 ($\pm$0.05, 1 standard error for 18 replicates). For CEO, the value was 57.9 ($\pm$1.6, 1 standard error for 18 replicates). No differences in the partition coefficients were observed when measured at initial headspace concentrations of 5 or 200 ppm. These values will be used for future studies designed to quantify the kinetics of CD and CEO metabolism in vitro.

4. Discussion

The proposed metabolic reactions of CD are presented in Fig. 9. This report is the first to identify CEO as an epoxide metabolite of CD. This finding extends the

results of an earlier study showing that hepatic microsomes produced a volatile metabolite of CD suggested to be an epoxide [13]. The oxidation of CD to CEO was evident in rodent and human liver microsomes (Figs. 1–3, Table 1), and most likely involves catabolism by CYP 2E1, shown by nearly complete in-vitro inhibition with 4-MP (Fig. 4). CYP 2E1 is an isozyme of cytochrome P450 oxidase involved in the metabolism of butadiene, isoprene, and other small organic molecules [37–39]. The inhibition of CD metabolism by 4-MP is consistent with its use to inhibit the in-vivo uptake of 1,3-butadiene [40]. However, the in-vivo studies with 4-MP and 1,3-butadiene showed partial inhibition, most likely because of the activity of other cytochrome P450 isozymes such as CYP 2A6 [41,42]. Therefore, it is possible that other cytochrome P450 isozymes in addition to CYP 2E1 will contribute to the metabolism of CD.

As part of metabolite identification, the relative amount of CEO present in microsomal incubations of each species was studied using 1-butanol as an internal standard for extraction and GC-MSD analysis. The results can be interpreted as suggesting that a greater amount of CEO was present in B6C3F1 mice and Fischer/344 rat liver microsomes followed by the Wistar rat, then human and hamsters (Table 1). Clearly, all five species formed CEO. As an epoxide metabolite, CEO would be expected to react with DNA and may be involved in the induction of tumors in laboratory rodents based on a recent study showing that CEO is mutagenic in *Salmonella typhimurium* [43]. Similar to 1,3-butadiene [44], understanding metabolism should help guide the planning and interpretation of any

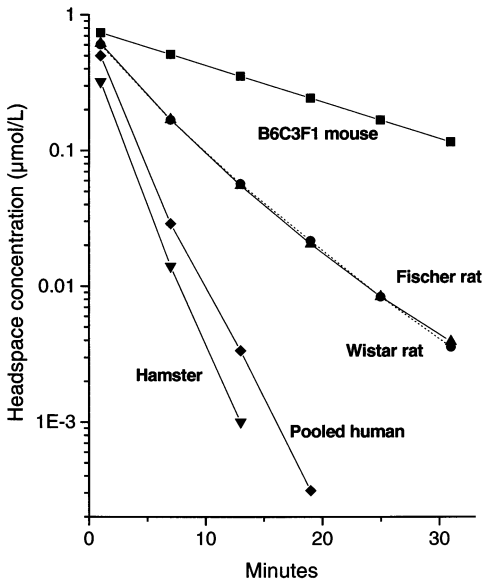

Fig. 6. Preliminary data showing the in-vitro uptake of CEO (100 ppm, 0.05 µmol) by enzymatic hydrolysis in liver microsomes containing 1 mg microsomal protein/ml.

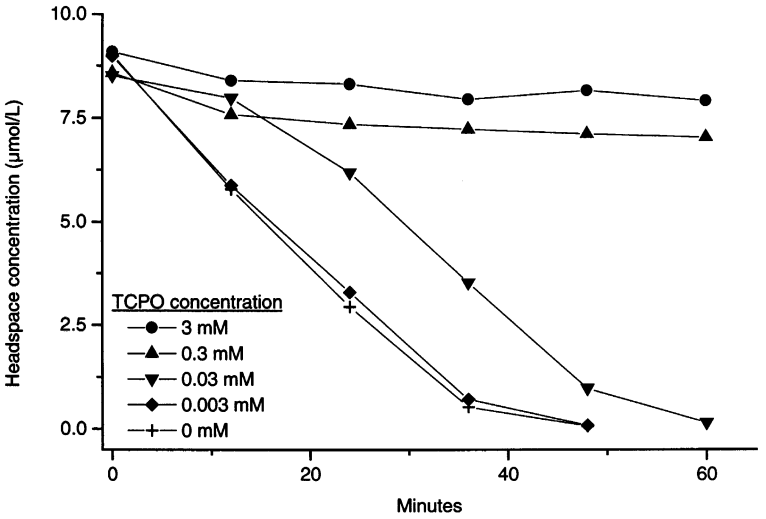

Fig. 7. Incubation of CEO (~2000 ppm, 0.9 µmol) with pooled human liver microsomes (0.5 mg microsomal of protein/ml) and TCPO (0–3 mM), a competitive inhibitor of epoxide hydrolase.

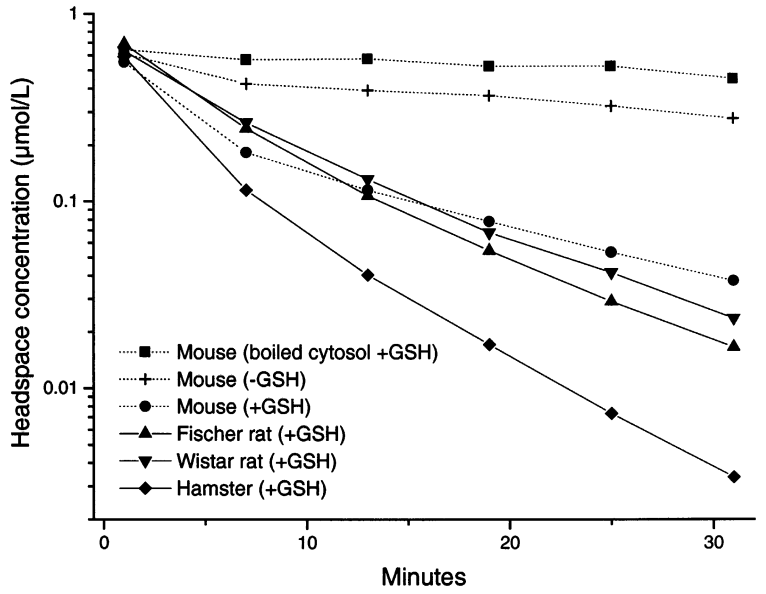

Fig. 8. Preliminary data showing glutathione conjugation of CEO (100 ppm, 0.05 µmol) in liver cytosol (1 mg of protein/ml).

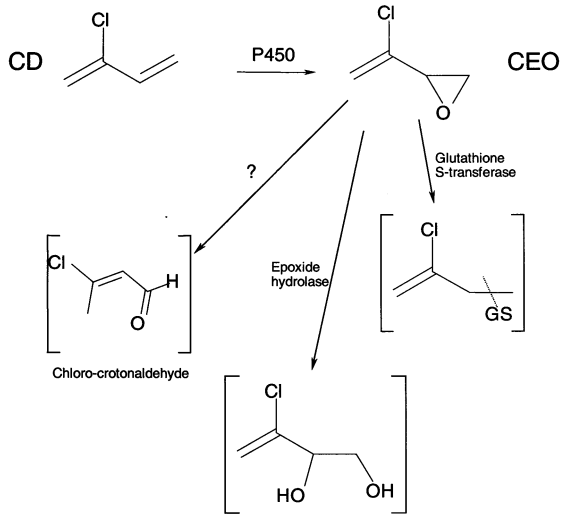

Fig. 9. Proposed metabolic pathway for β-chloroprene (CD) involving cytochrome P450 mediated oxidation to (1-chloroethenyl)oxirane (CEO), and further metabolism of CEO by epoxide hydrolase mediated hydrolysis, GSH conjugation by glutathione *S*-transferase, and possible formation of 3-chloro-2-butenal (chloro-crotonaldehyde).

future studies that might be designed to assess the role of DNA damage in the multi-site and species differences in tumorigenicity observed with CD.

Monitoring the time course of CD and CEO by GC/MSD has implications for designing further experiments to quantify the rate of CD oxidative metabolism. In the current study, CEO was sufficiently volatile to be quantified in the headspace despite having a liquid-to-air partition coefficient of 58. For example, the decline of CD was linked with the appearance of CEO in the vial headspace during an experiment using B6C3F1 mouse liver microsomes (Fig. 5). CEO was also detected in the vial headspace after incubation of CD with Wistar and Fischer rat microsomes but only after increasing the sample injection volume from 200 to 800 μl (data not shown). The uptake of CD from the headspace is linked to CEO formation, but it is also possible that more polar metabolites of CD were formed but not detected by diethyl ether extraction or headspace analysis in the current study. An underlying assumption is that a 1-to-1 stoichiometric relationship exists for conversion of the parent chemical to oxidized metabolite. Testing this assumption would involve the use of radiolabeled substrate, which has not been possible for technical reasons such as radiochemical instability. Despite this limitation, the analysis of CD oxidation rates without radiolabel appears to be a useful approach for interspecies comparisons as demonstrated for 1,3-butadiene [28]. The in-vitro studies with 1,3-butadiene (see review in Ref. [45]), showed that the rate of metabolism was faster in mice compared with rat or humans. The preliminary results of the current study suggest that this may also be the case for mice compared with the rat, hamster, or human. Further analytical optimization is under way to quantify CD oxidative metabolism in each of the five animals.

Further metabolism of CEO was observed in liver microsomes. For example, in mouse liver microsomes, the CEO concentration showed an initial increase over 10 min that was followed by a decline (Fig. 5), attributable to either epoxide hydrolase-mediated hydrolysis or further oxidative metabolism. Oxidative metabolism of CEO is being considered in further studies. The potential for species differences in the epoxide hydrolase-mediated hydrolysis of CEO was studied using an equal microsomal protein concentration for each of the five animals (Fig. 6). These preliminary results indicated that the hydrolysis of CEO in liver microsomes may be faster in hamsters $\sim$ humans $>$ rats $>$ mice. The dependence of the reaction on epoxide hydrolase was demonstrated in human microsomes using TCPO as a competitive inhibitor (Fig. 7) [46].

The interspecies differences in the susceptibility to CD-induced cancer between mice, rats, and hamsters could be related to the balance of CEO formation and detoxification. An additional reaction that may be important in the metabolism of CEO is GSH conjugation in cytosol. For example, the rate of conjugation of CEO with GSH in liver cytosol was slower in the mouse than the hamster (Fig. 8). The slower rate of detoxification of CEO by hydrolysis and GSH conjugation in the mouse compared with the hamster is consistent with the greater sensitivity of mice to CD-induced carcinogenicity than hamsters. The hamster was non-responsive in the chronic inhalation bioassay, whereas CD exposure induced tumors in a number of tissues in mouse [9,10]. Metabolism, in part, also may help interpret the observed difference in susceptibility for CD-induced tumorigenicity in Fischer and Wistar rats. The Fischer rat was more sensitive to CD-induced carcinogenicity than the Wistar rat. This study suggests that the relative amount of CEO present from CD oxidation and further CEO hydrolysis in the Fischer rat was greater than the Wistar rat (Table 1). CEO hydrolysis was similar between Fischer rat and Wistar rats. Conjugation with GSH appeared to be similar between the rats. Further research is needed to resolve these strain differences.

Generally, the preliminary in-vitro metabolism data presented here for mice, rats, and humans were consistent with published observation for the metabolism initial metabolism of 1,3-butadiene (BD) to epoxybutene (EB) [28,47]. BD is metabolized to EB and diepoxybutane (DEB) metabolites, and both epoxides exhibit species differences in the rate of reactions for hydrolysis by epoxide hydrolase and GSH conjugation by cytosolic GSH-S-transferase (see review in Ref. [45]). For EB, GSH conjugation was slower in humans than mice [28]. Isoprene, the 2-methyl analog of chloroprene and butadiene undergoes similar metabolism for which species differences in isoprene epoxidation also have been reported [48]. It is not yet known if CEO can be metabolized to a diepoxide.

5. Conclusions

This research establishes a foundation for understanding similarities and differences of CD metabolism among mice, rats, hamsters (animals used in the carcinogenicity studies) and humans. A GC-MSD analytical method was developed for the

analysis of CD metabolism, including oxidative metabolism of CD, and enzymatic hydrolysis and GSH conjugation of CEO. A key metabolite of CD in vitro appears to be CEO formed by a cytochrome P450-dependent reaction most likely mediated by CYP 2E1. The metabolism of CEO, like other epoxide analogs of butadiene and isoprene, involves enzyme-dependent hydrolysis and conjugation with GSH as detoxification reactions. The data indicate that the rates of CEO detoxification by epoxide hydrolase are fastest in hamsters ~ humans > rats > mice. The rates of CEO GSH conjugation in cytosol are hamster > rats ~ mice (human cytosol has not yet been tested). Further quantitative research is under way to establish kinetic parameters for these reactions in liver and lung, two target organs of CD-induced toxicity and carcinogenicity. The kinetic data will be used to parameterize a physiologically based toxicokinetic model (PBTK) for CD in rodents and develop a human PBTK model for application to estimating risk.

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